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The Adenovirus E3 RID Complex Protects Some Cultured Human T and B Lymphocytes from Fas-Induced Apoptosis

Adrienne L. McNees, C. T. Garnett, and Linda R. Gooding*

Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, Georgia 30322

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Human group C adenoviruses cause an acute infection in respiratory epithelia and establish a long-term or persistent infection, possibly in lymphocytes. The mechanism by which this persistence is maintained is unknown; however, it would require that persistently infected lymphocytes not be deleted. The adenovirus genome encodes proteins that prevent the immune system from eliminating the virus-infected cell, including the E3 receptor internalization and degradation (RID) complex. The RID complex prevents death of infected cells by blocking apoptosis initiated through death domain-containing receptors of the tumor necrosis factor receptor (TNFR) superfamily, including TNFR1 (L. R. Gooding, T. S. Ranheim, A. E. Tollefson, L. Aquino, P. Duerksen-Hughes, T. M. Horton, and W. S. Wold, J. Virol. 65:4114-4123, 1991), TNF-related apoptosis-inducing ligand receptors (TRAIL-R1 and -R2) (C. A. Benedict, P. S. Norris, T. I. Prigozy, J. L. Bodmer, J. A. Mahr, C. T. Garnett, F. Martinon, J. Tschopp, L. R. Gooding, and C. F. Ware, J. Biol. Chem. 276:3270-3278, 2001; A. E. Tollefson, K. Toth, K. Doronin, M. Kuppuswamy, O. A. Doronina, D. L. Lichtenstein, T. W. Hermiston, C. A. Smith, and W. S. Wold, J. Virol. 75:8875-8887, 2001), and Fas (J. Shisler, C. Yang, B. Walter, C. F. Ware, and L. R. Gooding, J. Virol. 71:8299-8306, 1997). Here, we test the ability of RID to protect human lymphocytes from apoptosis induced by ligation of Fas, a mechanism important for regulating lymphocyte populations. Using a retrovirus expressing RID to infect six human lymphocyte cell lines, we found that RID functions in the absence of other viral proteins to downregulate surface Fas on some, but not all, cell lines. Total cellular levels of Fas decrease as measured by Western blotting, and this loss of Fas correlates with protection from apoptosis induced by ligation of Fas in every cell line tested. Although in some cases, RID causes loss of only a fraction of surface Fas, the presence of RID completely blocks the immediate events downstream of Fas ligation (i.e., Fas-FADD association and caspase-8 cleavage) in susceptible cell lines. Nonetheless, the ability of RID to block Fas signaling is independent of the Fas signaling pathway used (type I or type II). Interestingly, among the four T-cell lines tested, RID caused loss of Fas in the two T-cell lines bearing a relatively immature phenotype, while having no activity in T cells with mature phenotypes. Collectively, these data suggest that RID functions to prevent apoptosis of some human lymphocytes by internalizing surface Fas receptors. It is possible that the expression of RID facilitates long-term infection by preventing Fas-mediated deletion of persistently infected lymphocytes.

Group C (types 1, 2, 5, and 6) adenoviruses (Ads) are ubiquitous in the human population and typically infect the epithelium of the upper respiratory tract (reviewed in reference 24). As with most DNA viruses, Ads encode proteins that function to counteract host antiviral responses that could limit successful infection (reviewed in reference 18). Several viral products serve to block ligand-induced cell death pathways. A number of investigators have demonstrated inhibition of Fas-, tumor necrosis factor receptor 1 (TNFR1)-, and/or TNF-related apoptosis-inducing ligand receptor 1 (TRAIL-R1)-triggered cell death by three separate viral mechanisms: the E1B 19K, E3 14.7K, and the E3 RID (receptor internalization and degradation) complex (18).

The RID complex is composed of two forms of the protein with a molecular weight of 10,400 (10.4K protein) (29) and the14.5K protein (28), which form a heterotrimer that localizes to the plasma membrane (26, 29). The first activity ascribed to RID was induction of loss of the epidermal growth factor receptor (EGFR) from the surface of virus-infected cells (4). RID was later found to block apoptosis induced by both tumor necrosis factor (TNF) (12) and Fas (25). Protection from Fas-mediated killing correlates with the removal of the Fas receptors from the surface of infected cells and their degradation in lysosomes (8, 25, 27).

The presence of redundant mechanisms to block apoptosis signaled through death receptors suggests that blockade of ligand-triggered apoptosis is a high priority for some stage in the virus life cycle, which includes both an acute phase and a persistent phase. Epidemiological studies have shown that Ads persist for several years following primary infection, with intermittent shedding of virus in the feces (9, 10). The cells harboring the persistent virus have not been identified, but several lines of evidence suggest they are lymphocytes. In early studies, group C Ads were isolated from lymphocytes derived from tonsils and adenoids (31). Also, viral DNA has been recovered from tonsils in the absence of viral replication after long-term tissue culture (20). Recent studies in our laboratory have localized group C Ad DNA to human tonsillar T lymphocytes in the absence of active virus replication (11a).

Blockade of Fas- or TNFR1-mediated death could prolong the life of an acutely infected epithelial cell and optimize virus

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Emory University School of Medicine, 3108 Rollins Research Center, 1510 Clifton Rd., Atlanta, GA 30322. Phone: (404) 727-5948. Fax: (404) 727-0293. E-mail: gooding@microbio.emory.edu.

production in the face of host immune and inflammatory defenses. However, blockade of these pathways in persistently or latently infected lymphocytes could have even more profound consequences. Ligand-induced cell death via death receptors is required for elimination of self-reactive T cells in the periphery and contributes to downregulation of T-lymphocyte populations at the end of an immune response (16). Fas is also required for death of peripheral B cells during affinity maturation in lymphoid follicles (16). In addition, some cytotoxic T lymphocytes use Fas-L to kill target cells (1). Humans and mice lacking Fas or Fas-L develop lymphadenopathy and autoimmunity with defects in both the T- and B-cell compartments (19). Persistent Ads expressing the E3 proteins may block Fas and TNFR1 signaling, permitting the infected cell to temporarily or permanently avoid cell death.

Given the potential for Ad to persist in lymphocytes and the likely requirement to block apoptosis of persistently infected cells, the ability of RID to block Fas-triggered apoptosis in human lymphocytes was investigated by using a series of human T- and B-lymphocyte cell lines. A retroviral vector was used to transduce cells to establish stable RID expression.

MATERIALS AND METHODS

Cell lines. SKW6.4, Jurkat, CEM, and HuT78 cell lines were obtained from the American Type Culture Collection. BJAB cells were obtained from Carl Ware (La Jolla Institute for Allergy and Immunology). KE-37 cells were obtained from Periasamy Selvaraj (Emory University). All lymphocyte cell lines were grown in RPMI medium with 10% fetal calf serum (HyClone, Logan, Utah) and 10 mM glutamine. The packaging cell line BOSC23 (21) for retrovirus production was obtained from Joshy Jacob (Emory University) and was grown in Dulbecco's modified Eagle medium with 10% fetal calf serum and 10 mM glutamine.

Antibodies. Antibodies were obtained from the following sources. Anti-Fas antibodies IPO-4 and APO-1-3 were purchased from Kamiya Biomedical Company (Seattle, Wash.), anti-Fas antibodies C-20 and B-10 were purchased from Santa Cruz Biotechnology, the mouse anti-FADD antibody was purchased from Transduction Laboratories (Lexington, Ky.), and the mouse anti-caspase-8 antibodies recognizing the enzyme proform (66231A) or the processed forms (9746) were purchased from BD Biosciences Pharmingen (San Diego, Calif.) or from Cell Signaling Technology (Beverly, Mass.), respectively. The anti-adaptin-α (AP-2) was purchased from BD Transduction Labs. The secondary antibody-horseradish peroxidase conjugates used for Western blotting were anti-mouse immunoglobulin (Ig) or antirabbit Ig and were purchased from Amersham. The antibodies specific for the E3-10.4K and -14.5K proteins were made by immunizing rabbits with the following peptides coupled to keyhole limpet hemocyanin: for anti-10.4K, oligonucleotide QFIDWVCVRIAYLRHHP QYRDRTIADLLRIL (corresponding to residues 60 to 91 of the Ad2 protein); and for anti-14.5K, oligonucleotide GGFVPANQPRPPSPTPTEISYFNLTGG DD (corresponding to residues 105 to 132 of the Ad5 protein). Both antibodies were affinity purified from rabbit polyclonal antiserum by coupling the immunizing peptides to HiTrap NHS Sepharose columns (Amersham Pharmacia Biotech) followed by affinity purification from the rabbit serum according to the manufacturer's instructions.

Retrovirus production and transduction. The Ad2 E3 10.4K and 14.5K sequences were excised in tandem from the Ad2 *Eco*RV plasmid (15) and cloned by PCR into the *Bgl*II site downstream of the long terminal repeat in the murine stem cell virus (MSCV) plasmid (pMSCV) (Clonetech). The following PCR primers were used: forward primer 5′-TCCAGATCTCTCGAGCCACCATGA TTCCTCGAGTTCTTATA-3′ and reverse primer 5′-GCTAGATCTGCGGC CGCTATCAGTCATCTCCACCTGTCAA-3′. PCR was carried out with *Pfu* polymerase for 30 cycles, and the resulting PCR product of approximately 680 bp was agarose gel purified. The cleaned PCR product and the MSCV vector were digested with *Bgl*II from New England Biolabs (NEB). The digested MSCV and digested PCR-treated RID were ligated together with NEB ligase overnight at 4°C. The ligation reaction was then transformed into competent JM109 cells (Stratagene). The resulting pMSCV-RID plasmid insert was sequenced and confirmed to be correct.

By the calcium phosphate method, the pMSCV control plasmid or the pMSCV-RID plasmid was transfected into the packaging cell line BOSC23, which stably expresses the structural genes *gag*, *pol*, and *env* necessary for particle formation and replication. For use in human cells, the retrovirus was pseudotyped with the vesicular stomatitis virus G protein (VSV-G) by cotransfection of a plasmid encoding this gene (2). Two days posttransfection, the virus-containing supernatants were harvested and then filtered with a 0.45-μm-pore-diameter syringe filter, and Polybrene was added to the viral stock to a final concentration of 8 μg/ml. To transduce human lymphocyte cell lines, 1 ml of virus stock was added to 10^6 cells in1 ml of RPMI complete medium, and the mixture was centrifuged for 45 min at 25°C and 500 × *g*. Puromycin (4 μg/ml; Sigma) was added 48 h posttransduction to select for cells expressing the puromycin resistance gene encoded in the MSCV plasmid.

Detection of cell surface Fas. Cells were stained for surface Fas with the IPO-4 antibody (Kamiya) or the IgM isotype-matched control antibody (Caltag Laboratories) and goat anti-mouse IgM–allophycocyanin (Caltag Laboratories). Cells were analyzed on a FACS-Calibur fluorescence-activated cell sorter (FACS) flow cytometer (Becton Dickinson) with Cellquest software.

Assay of anti-Fas-induced apoptosis. Cells were incubated at a density of 10^6 cells per ml with 1 μg of IPO-4 per ml (Kamiya) and 1 μg of cycloheximide (CHX) per ml (Sigma). At the indicated times, cells were harvested and stained with annexin V-PE (Pharmingen) according to the manufacturer's instructions and analyzed with the FACS-Calibur. The data are presented as the percentage of cells staining positively for annexin-V PE staining. No staining backgrounds have been subtracted.

Western blots. Cell lysates were made by washing cells in phosphate-buffered saline (PBS) and resuspending them in either NP-40 lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40 [Sigma], 0.1 M dithiothreitol, and protease inhibitor cocktail) (Roche) or sodium dodecyl sulfate (SDS) lysis buffer from Bio-Rad (62.5 mM Tris-HCl [pH 6.8], 2% SDS, 25% glycerol, 0.01% bromophenol blue) at a density of 10^7 cells per ml. Western blots for FADD and caspase-8 were performed by resolving 100 μg of lysate from each cell line on a 12.5% Bio-Rad Criterion gel. Lysates to be blotted with anti-Fas were resolved by 8 to 18% polyacrylamide gradient SDS-polyacrylamide gel electrophoresis (PAGE). In all cases, the proteins were transferred to nitrocellulose (Osmonics, Inc.) by semidry transfer. The membranes were blocked in 3% bovine serum albumin in Tris-buffered saline (TBS)-Tween (TBS plus 0.05% Tween 20) for 1 h at 25°C. Both the primary and secondary antibodies were incubated for 1 h at 25°C. Blots were developed with Renaissance chemiluminescence reagents (NEN) and Kodak Biomax film.

The processing of caspase-8 was determined by treating cells as described for the induction of apoptosis. Lysates equivalent to 10^6 cells were resolved on a 4 to 20% Bio-Rad Criterion gel and blotted as described above.

DISC analysis. Formation of the death-inducing signaling complex (DISC) was assayed essentially as described by Scaffidi et al. (23). Cells at a density of 10^7 per ml were treated with 2 μg of APO-1-3 per ml for 5 min at 37°C. Cells were washed once in PBS and lysed in NP-40 lysis buffer. Unstimulated control cells were first lysed, and then APO-1-3 was added to the lysates. The DISC was precipitated by incubating lysates with protein A-agarose beads (Santa Cruz Biotechnology) for 1 h at 4°C. The beads were washed three times with a total of 15 volumes of lysis buffer. The DISC components were separated on a 12.5% Bio-Rad Criterion gel, and Western blotting was performed as described above.

RESULTS

Surface Fas expression and sensitivity of human lymphocyte cell lines to Fas-mediated apoptosis. It has been suggested that lymphocytes are the reservoir for a persistent or latent Ad infection, and identification of the cell type that harbors persistent virus is currently under investigation in our laboratory. We and others have shown that RID expressed in acute viral infection functions to protect epithelial cells from Fas-induced apoptosis (8, 25, 27), a mechanism important in regulating lymphocyte populations (reviewed in reference 16). The goal of this study was to determine if RID functions in human lymphocyte cell lines to block Fas-mediated cell death, a function that may contribute to the maintenance of a persistent infection in lymphocytes. RID's function was evaluated in a panel of lymphocyte cell lines, including two B-cell lines

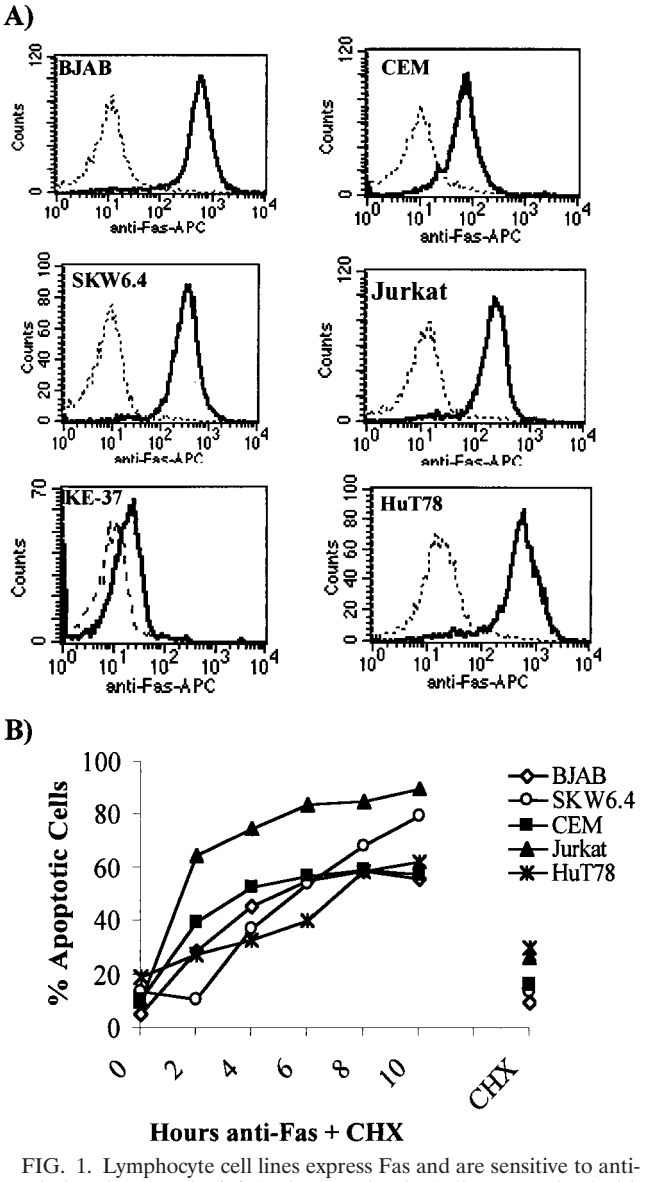

FIG. 1. Lymphocyte cell lines express Fas and are sensitive to anti-Fas-induced apoptosis. (A) Surface Fas levels. Cells were stained with an isotype control (dashed lines) or with anti-Fas antibody (IPO-4) (solid lines), incubated with goat anti-mouse IgM–allophycocyanin, and analyzed with a FACS-Calibur flow cytometer and Cellquest software. (B) Sensitivity of lymphocyte cell lines to anti-Fas-induced apoptosis. Cells were treated for the indicated times with anti-Fas (IPO-4) and CHX, stained with annexin V-PE, and analyzed by flow cytometry. The points marked CHX represent apoptosis in cells incubated for 10 h in CHX in the absence of anti-Fas. The data shown are representative of repeated experiments.

(BJAB and SKW6.4) and four T-cell lines (Jurkat, CEM, HuT78, and KE-37). The suitability of these cell lines for this study was first confirmed by measuring surface Fas expression and the sensitivity of these cells to anti-Fas-induced apoptosis. As shown in Fig. 1A, five of the cell lines expressed high levels of the surface Fas receptor. The sixth line, KE-37, expresses very little surface Fas and is resistant to Fas-triggered apoptosis (data not shown). Additionally, all cell lines are sensitive to apoptosis induced by treatment with anti-Fas (Fig. 1B). CHX sensitizes cells to apoptosis (11), and CHX was included in the

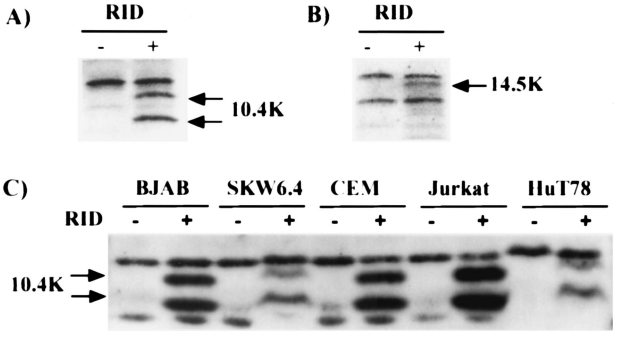

FIG. 2. Expression of RID by MSCV. NP-40 lysates (100 μg of protein) from the BOSC23 packaging cells transfected with either pMSCV control or with pMSCV-RID were resolved by 8 to 8% polyacrylamide gradient SDS-PAGE, followed by Western blots for 10.4K (A) or 14.5K (B) protein. (C) NP-40 lysates (100 μg of protein) from transduced lymphocyte cell lines were resolved by 8 to 18% gradient SDS-PAGE, followed by Western blotting for 10.4K.

apoptosis induction protocol used here. As shown in Fig. 1B, CHX alone accounts for only a small fraction of cell death observed over the 10-h time course.

Retroviral RID expression. MSCV was used to make RID-expressing lymphocyte lines. Retrovirus stocks were produced by transfection of either the control plasmid, pMSCV, or the RID-expressing plasmid, pMSCV-RID, into the BOSC23 packaging cell lines. Two days posttransfection, the retrovirus-containing supernatant was collected, and lysates were made from the transfected BOSC23 cells. Expression of the RID proteins in the packaging cell line was confirmed by Western blotting. As shown in Fig. 2A and B, both RID (10.4K and 14.5K) subunits were expressed during retroviral production. The anti-14.5K antibody was made to the Ad5 version of the protein, which contains five amino acid differences compared with the Ad2 protein used here. The antibody reacts specifically, but relatively weakly, with the Ad2 14.5K protein (data not shown). Because both proteins are expressed from a single plasmid, further analysis was performed with only the anti-10.4K antiserum.

The retroviral stocks were then used to make stable cell lines with the six human lymphocyte cell lines illustrated in Fig. 1. All cell lines were infected with either MSCV as a control or with MSCV expressing RID. Following puromycin selection, RID expression in the lymphocyte cell lines was confirmed by Western blotting for the 10.4K RID subunit. As shown in Fig. 2C, RID is expressed in all cell lines transduced with MSCV-RID and is absent in cells transduced with the control retrovirus.

Type I and type II Fas signaling lymphocytes. Two Fas signaling pathways have been described (22). Type I signaling is characterized by rapid formation of the death-inducing signaling complex (DISC) following Fas receptor ligation. This includes recruitment of Fas-associated death domain (FADD) and caspase-8 to the Fas receptor. Type II cells show reduced DISC formation, although both cell types express similar levels of the DISC components. Caspase-8 activation occurs at the level of the DISC in type I cells and follows activation of caspase-3 in type II cells, although the kinetics of apoptosis are similar for both pathways. The designation as a type I or type II signaling cell is made by treating cells with anti-Fas to induce

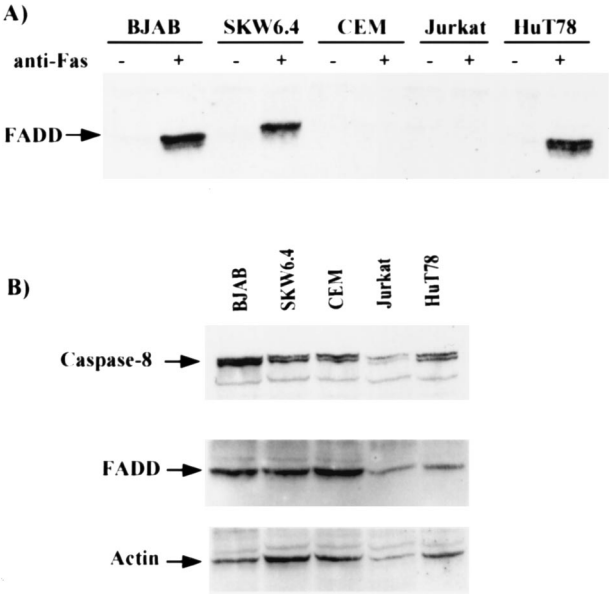

FIG. 3. FADD and caspase-8 expression and DISC formation in lymphocyte cell lines. (A) DISC immunoprecipitation from lymphocyte cell lines. A total of 10^7 cells were either untreated or treated with anti-Fas (APO-1-3) for 5 min to induce DISC formation as described in Materials and Methods. Complexes were dissolved in NP-40 lysis buffer, precipitated with protein A-agarose beads, and resolved by SDS-PAGE (12.5% polyacrylamide), followed by Western blotting for FADD. (B) NP-40 lysates (100 μg of protein per lane) from untreated lymphocyte cell lines were resolved by SDS-PAGE (12.5% polyacrylamide) followed by sequential Western blots for FADD, caspase-8 (Pharmingen), and actin. A single membrane was used for all of these blots.

complex formation, then precipitating these complexes and resolving them by SDS-PAGE. FADD and caspase-8 coprecipitate with Fas in type I signaling cells, but not in type II cells.

Given the previously characterized function of RID to internalize the Fas receptor, it seemed a likely possibility that RID may act at the plasma membrane to block recruitment of DISC components to the Fas receptor. Therefore, we hypothesized that RID may function to block apoptosis only in type I Fas signaling cells by disrupting DISC formation. RID's function was evaluated in both type I and type II Fas signaling cells in order to test this hypothesis. By the method of Scaffidi et al. (23), the lymphocyte cell lines used here were characterized as type I or type II by measuring DISC formation following Fas receptor ligation. KE-37 cells, which are not Fas sensitive relative to the other cell lines, were omitted from this analysis. As shown in Fig. 3A, FADD was not recruited to Fas in any cell lines without receptor stimulation. FADD did coimmunoprecipitate with Fas in the BJAB, SKW6.4, and HuT78 cell lines following stimulation of the Fas receptor, indicating a type I Fas signaling pathway. In contrast, FADD did not bind Fas in the Jurkat and CEM cell lines following Fas ligation (Fig. 3A). Thus, these cells use type II Fas signaling. These results agree with those of Scaffidi et al., who have shown that SKW6.4 and HuT78 are type I cells and Jurkat is a type II cell (22).

The relative abundance of DISC proteins was evaluated by measuring expression of FADD and caspase-8 by Western blotting (Fig. 3B). The levels of both proteins are similar in all of the cell lines when corrected for the actin loading control. These results again agree with those of Scaffidi et al. that the signaling pathway used is not determined by the expression levels of DISC components (22).

RID-mediated protection from Fas-induced apoptosis correlates with loss of surface Fas. RID functions to internalize and degrade Fas receptors during the course of lytic infection in epithelial cells (8, 25, 27). However, RID function is cell type dependent. The 10.4K and 14.5K proteins encoded by the E3 region of Ad function together to protect many but not all mouse cell lines against lysis by TNF (12). Therefore, the ability of RID to cause loss of surface Fas and/or block Fas-mediated apoptosis in lymphocytes was tested directly with six retrovirus-transduced human cell lines. Infection of the six human lymphocyte cell lines with either MSCV as a control or MSCV expressing RID did not appear to change the growth properties or morphology of the cells. These cells were tested for surface Fas levels and sensitivity to anti-Fas-induced apoptosis. As shown in Fig. 4B, the MSCV vector alone had no effect on surface Fas levels. However, stable expression of RID mediated downregulation of surface Fas on BJAB, SKW6.4, and CEM cells. In each of these three cell lines, some Fas expression remained in the RID-transduced population. In BJAB and CEM cells, a subpopulation of cells expressing the same levels of Fas as control cells was seen among RID-positive cells. In SKW6.4, the level of Fas was diminished, but only to a level higher than that of MSCV-transduced control CEM cells. Although KE-37 cells express low levels of surface Fas, RID did function to reduce surface Fas expression. In contrast, RID expression had no measurable effect on surface Fas levels on HuT78 and Jurkat cell lines.

The Fas downregulation correlated with protection from Fas-induced apoptosis, even in cells where loss of surface Fas is not complete. BJAB, SKW6.4, and CEM cell lines expressing RID were protected from anti-Fas-induced apoptosis (Fig. 4A). RID expression had no protective effect in Jurkat and HuT78 cell lines. KE-37 cells have a reduced sensitivity to anti-Fas-mediated apoptosis relative to the other cell lines tested, probably due to low levels of the receptor. However, the presence of RID did seem to reduce the percentage of apoptotic cells following anti-Fas treatment. Collectively, these data show that RID functions in the absence of other viral proteins to mediate Fas downregulation and block Fas-induced cell death in human lymphocyte cell lines. These functions of RID do not depend upon lymphocyte lineage, since both B cells (BJAB and SKW6.4) and T cells (CEM) are protected. Neither does protection depend upon the use of type I or type II Fas signaling pathways, because protection is seen in both type I Fas signaling cells (BJAB and SKW6.4) and type II Fas signaling cells (CEM).

It is technically possible that, in the cell lines in which RID apparently did not function (Jurkat and HuT78), only a small percentage of cells express RID. An alternative approach was used to confirm the data obtained with the retrovirus-transduced cell lines. Lymphocyte cell lines were infected with wild-type Ad (*rec*700) or a deletion mutant that lacks only the RID proteins (*dl*799). Live infected cells were first stained for surface Fas expression, permeabilized, and then stained for the intracellular hexon. Gating on hexon-positive cells and analysis of the surface levels of Fas revealed that RID functioned to

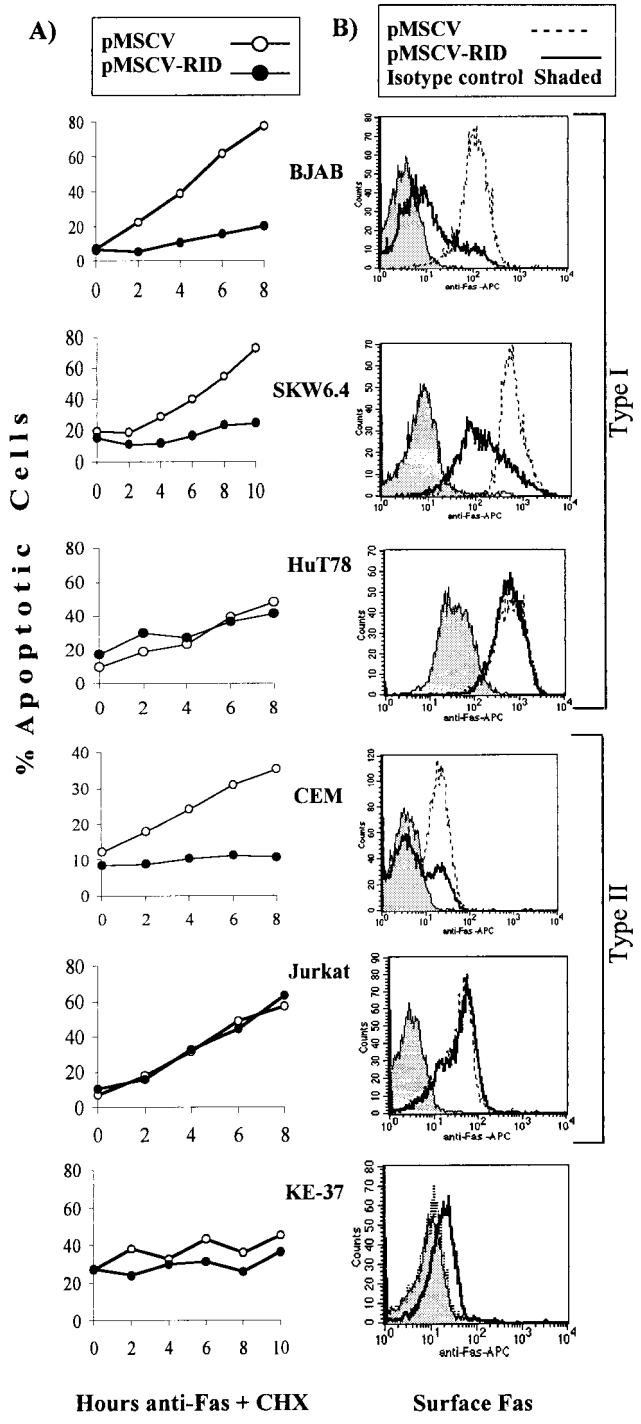

FIG. 4. Protection from anti-Fas-induced apoptosis correlates with loss of surface Fas receptors. (A) RID protects BJAB, SKW6.4, and CEM cells from anti-Fas-induced apoptosis. Cells were transduced with either the MSCV control or MSCV-RID as indicated. Apoptosis was measured by annexin V-PE staining according to the apoptosis induction protocol described in Materials in Methods. The data shown are representative of repeated experiments. Note the change in scale on the *y* axis for the CEM plot. (B) RID expression causes downregulation of Fas on BJAB, SKW6.4, CEM, and KE-37 cells. Cells transduced with either the MSCV control or MSCV-RID were stained with an isotype control or with anti-Fas (IPO-4) as indicated and analyzed by flow cytometry.

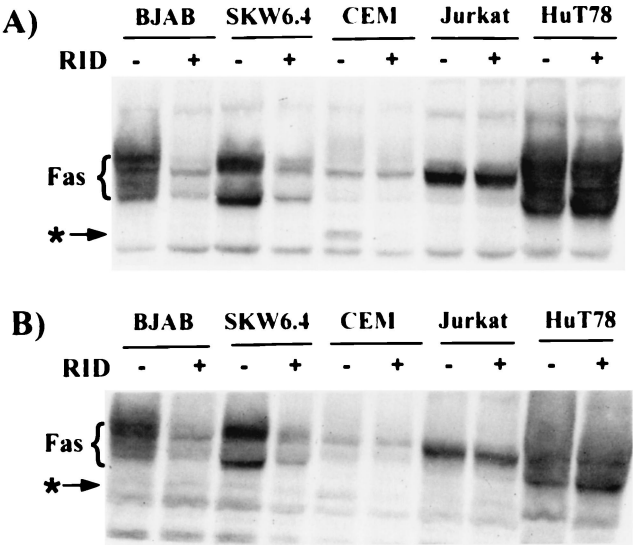

FIG. 5. RID mediates a decrease in total cellular Fas levels in some lymphocyte cell lines. In both blots, each lane contains lysates from 2 $\times$ 10^6 cells. Proteins were resolved by 8 to 18% polyacrylamide gradient SDS-PAGE, followed by Western blotting for Fas (anti-Fas; Santa Cruz Biotechnology). Lysates were made with NP-40 lysis buffer (A) or with SDS lysis buffer (B) as described in Materials and Methods. An asterisk indicates that only one small isoform of Fas is affected by the presence of RID in the CEM cell line.

downregulate surface Fas in CEM and BJAB cells, but not on Jurkat or HuT78 cells (data not shown). Thus, specific protein staining of infected lymphocytes confirms the observations made with the cell lines stably expressing RID.

Fas is degraded in lymphocyte cell lines. We have previously published that in the context of a viral infection RID induces internalization of Fas, but the intracellular pools of Fas protein remain high, as measured by confocal microscopy (25). In contrast, others have shown that the Fas protein is degraded following RID expression (8, 27). In this study, we reexamined this question by evaluating the total levels of Fas in the presence of RID expressed in a steady state in the retrovirus-transduced cell lines. Cells were lysed with NP-40, a nonionic detergent that does not disrupt nuclei and other cellular compartments, and the insoluble material was removed by centrifugation. These lysates were resolved by SDS-PAGE followed by Western blotting for the Fas proteins. As shown in Fig. 5A, Fas levels were reduced in BJAB and SKW6.4 in the presence of RID. Expression of only one isoform of Fas was reduced in the RID-expressing CEM cells. Total Fas levels appeared equal in Jurkat and Hut78 cell lines in the presence and absence of RID.

To determine if RID directs Fas to an insoluble cellular compartment, parallel cell lysates were made with SDS, an ionic detergent that disrupts intracellular membranes, releasing contents that would be insoluble in the NP-40 lysate. These total cellular lysates were resolved by SDS-PAGE followed by Western blotting, and the results are shown in Fig. 5B. The pattern of Fas expression was the same with either NP-40 or SDS lysis buffers. Total Fas levels were reduced in RID-expressing BJAB and SKW6.4 cells, and only one form of Fas was affected by RID in CEM cells (Fig. 5B). In these total

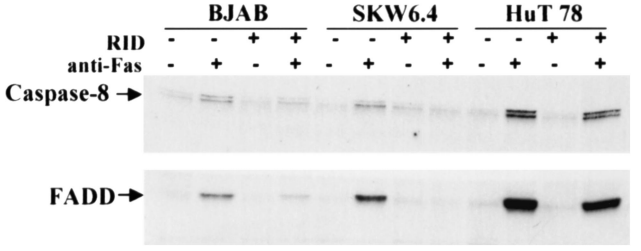

FIG. 6. The presence of RID prevents the recruitment of FADD and caspase-8 to Fas in some type I Fas signaling cells. MSCV control- and MSCV-RID-transduced cell lines were either untreated or treated with anti-Fas (APO-1-3) for 5 min to induce DISC formation as described in Materials and Methods. Complexes were dissolved in NP-40 lysis buffer, precipitated with protein A-agarose beads, and resolved by SDS-PAGE (12.5% polyacrylamide), followed by Western blotting for caspase-8 (Pharmingen) and FADD.

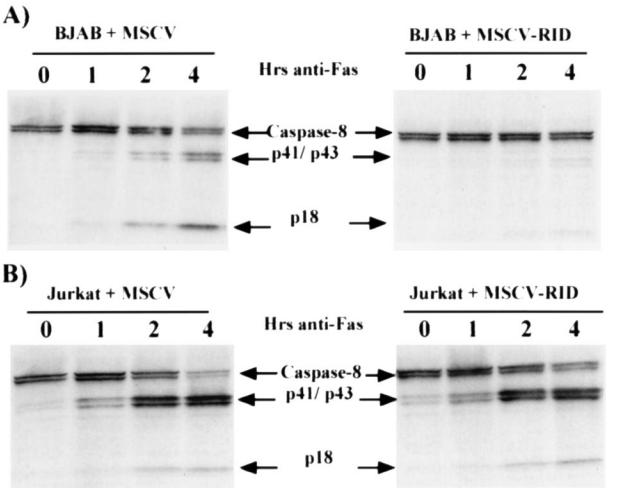

FIG. 7. Caspase-8 activation is blocked by RID in some lymphocyte cell lines. BJAB (A) and Jurkat (B) cells were transduced with either the MSCV control or with MSCV-RID, as indicated on the figure and were treated with anti-Fas (IPO-4) to induce apoptosis as described in Materials and Methods. NP-40 lysates were made at the indicated times and resolved by 4 to 20% polyacrylamide gradient SDS-PAGE, followed by Western blotting for caspase-8 (Cell Signaling Technology). Each lane contains lysates from 10^6 cells. Western blots show the caspase-8 proform, intermediate cleavage products (p41/p43), and the active form (p18).

cellular lysates, Fas levels were undiminished in Jurkat and Hut78 cells expressing RID. These data rule out the possibility that RID causes Fas to accumulate in an insoluble intracellular compartment. These data correlate with the results shown in Fig. 4; Fas is internalized on the same cell lines in which it is degraded.

RID functions above the level of the DISC to block caspase-8 activation. The findings described above establish that RID's functions—downregulation of Fas and protection from Fas-induced apoptosis—are independent of type I or type II Fas signaling. This suggests that the sole RID function is to remove Fas from the cell surface, obviating the necessity of blocking both signaling pathways. However, the fact that some RID-transduced cell lines resist killing while expressing significant levels of Fas on their surface suggests that there may be additional mechanisms by which RID blocks Fas signaling. To determine the site at which the blockade occurs, the type I cells (BJAB, SKW6.4, and HuT78) were analyzed for the earliest events in DISC formation (association of FADD and caspase-8) by coimmunoprecipitation with Fas. As shown in Fig. 6, the presence of RID blocked the ligation-induced recruitment of both DISC components to the Fas receptor in the RID-sensitive lines, BJAB and SKW6.4, but not in RID-insensitive HuT78. Thus, although some Fas remains on the surface of SKW6.4 cells, it cannot bind the upstream DISC components following Fas ligation. The small amount of DISC formation seen in RID-transduced BJAB cells may derive from the small Fas-expressing population seen in Fig. 4B.

The first enzymatic event following Fas ligation is the cleavage and activation of capase-8, and blockade of this step prevents Fas-induced apoptosis. As expected, the processing of the proform of caspase-8 to the active form was blocked in RID-expressing BJAB cells, but was unaffected in RID-resistant Jurkat cells (Fig. 7). Additionally, caspase-8 activation was blocked in CEM and SKW6.4 cells in the presence of RID (data not shown).

DISCUSSION

The findings presented here demonstrate that RID can act independently of other viral proteins to mediate the internalization and degradation of Fas in human lymphocyte cell lines. The loss of the surface Fas receptor correlates with protection from anti-Fas-induced apoptosis. Cells in which surface Fas is decreased as a consequence of RID expression (BJAB, SKW6.4, and CEM) are protected from Fas-induced apoptosis, while cells in which surface Fas is not decreased (Jurkat and HuT78) are not protected. Recent studies have also shown that RID, expressed either during virus infection or transiently in combination with the E3 6.7K protein, mediates the internalization of TRAIL-R1 and -R2 (2, 30). As in our study with the Fas receptor, loss of the surface TRAIL receptors correlates with protection from apoptosis triggered through the receptor, suggesting that one mechanism by which RID blocks apoptosis is by removing surface death receptors. However, RID is capable of at least one other antiapoptotic mechanism, as illustrated by its blockade of TNFR1 signaling. RID acts to block TNF-induced apoptosis, which is mediated through TNFR1, without decreasing surface TNFR1 levels (2, 25), suggesting RID also functions at a postreceptor binding step.

One key question, of course, is why RID protects some lymphocyte cell lines but not others from Fas-induced apoptosis. Lymphocyte cell lines use one of two characterized Fas signaling pathways (22). Thus, one hypothesis predicts that RID could interfere with one pathway, but not the other. This was not borne out experimentally. RID protects both type I (BJAB and SKW6.4) and type II (CEM) Fas signaling cell types from Fas-induced killing. The two cell lines that are not protected are one type I line (HuT78) and one type II line (Jurkat). Thus, RID's functions are independent of the formation of a signaling complex, the criterion for type I signaling. This suggests that RID functions above the level of DISC formation and blocks apoptotic signaling by removing active receptors, despite the fact that significant levels of Fas remain

on some RID-transduced cells. Also, the fact that RID functions in the absence (CEM) or presence (BJAB and SKW6.4) of a DISC suggests that RID's function does not depend on interactions with other molecules involved in Fas signaling, including FADD and caspase-8.

RID induces internalization of an array of surface receptors including Fas (8, 25, 27), EGFR (4), TRAIL-R1 and -R2 (2, 30), the insulin receptor, and the insulin-like growth factor receptor I (17). The cytoplasmic regions of these receptors contain no obvious common domain to which the RID proteins might bind. Thus, RID may bind directly to molecules involved in protein sorting, rather than to the receptors themselves, to cause receptor internalization, and these sorting proteins vary from one cell line to another. The cytoplasmic domains of RID contain two putative protein-sorting motifs that could mediate interactions with clathrin adaptor protein (AP) complexes (reviewed in reference 13). The YXXO motif (where Y is tyrosine, X is any amino acid, and O is a bulky hydrophobic amino acid) is present in the cytoplasmic domain of 14.5K, and a leucine-based lysosomal sorting signal (LL) is present in the cytoplasmic domain of 10.4K. The dileucine motif in the cytoplasmic tail of the EGFR is required for the internalization of this receptor mediated by 10.4K, suggesting that interactions between these homologous motifs may direct receptors to lysosomal compartments (6).

The AP-2 complex mediates clathrin-dependent vesicular transport from the plasma membrane to endosomes by binding to cytoplasmic tails of receptors via a dileucine-based sorting motif (13), the domain implicated in the interaction between 10.4K and the EGFR (6). All lymphocyte cell lines tested in this study express equal amounts of AP-2, as measured by Western blotting for the α subunit (data not shown). Thus, the absence of RID's functions in Jurkat and HuT78 cells is not due to the absence of proteins that probably mediate the interaction of RID with the Fas receptor.

Several studies have described and classified lymphocyte cell lines as immature or mature based on imunophenotyping and T-cell receptor gene rearrangements (3, 7, 32). According to these authors, two of the T-cell lines used in this study bear the characteristics of immature pre-T or cortical T cells (CEM and KE-37), while the other two resemble mature T cells (Jurkat and HuT78), and we have confirmed these designations (data not shown). Interestingly, RID functions to downregulate Fas in the immature T cells, but not in the mature T cells (Fig. 4). One might speculate that, if indeed the virus persists in T cells, initial infection of an immature T cell might require protection from ligand-induced cell death, while loss of function in an activated cell might facilitate virus release.

The data described here also confirm that RID functions independently to mediate the degradation of Fas proteins following their removal from the cell surface. In an earlier study using virus-infected cells, we did not observe a decrease in total cellular levels of Fas (25). This important difference may reflect a variation in RID's function between the steady-state expression achieved by retroviral transduction and the transient expression seen in a viral infection. Perhaps in acute infection, downregulation of surface Fas is not accompanied by rapid degradation due to inhibition of cellular activities in lytic infection. The results reported here do agree with others showing that the total levels of Fas proteins decrease with RID expression (8, 27). An interesting observation made in this study is that only one isoform of Fas in the CEM cell line is targeted by RID for internalization and degradation. The Fas protein undergoes alternative splicing to generate several isoforms, and variations are seen among cells from different donors (5, 14). Perhaps the Fas splice variants that are not targeted for degradation by RID are present in intracellular stores and do not interact with RID at the plasma membrane. Further characterization of this Fas isoform may provide insight into how RID interacts with surface receptors. The FACS data indicate that a small percentage of RID-expressing CEM cells remain Fas positive, suggesting that there may be some heterogeneity of Fas isoform expression in this cell population.

This study demonstrates that the E3 RID complex protects lymphocytes from apoptosis induced by signaling through the Fas receptor and that this protection may depend upon the stage of differentiation of the lymphocyte. This in turn suggests that in natural Ad infection, persistently or latently infected lymphocytes may enjoy preferential survival due to blockade of normal apoptotic signals.

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